Secretory immune responses in the mouse vagina after parenteral or intravaginal immunization with an immunostimulating complex (ISCOM)

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Immunostimulating complexes (ISCOMs) are subunit vaccines that are particularly effective in producing immunity against systemic viral infections, but their effectiveness against mucosal infections has received little attention. To study their ability to produce mucosal immune responses in the female reproductive tract, a model ISCOM was prepared containing sheep erythrocyte membrane proteins, and anti-erythrocyte IgA and IgG titres in mouse vaginal washings were measured after immunization at parenteral or local mucosal sites. The ISCOM was prepared by a modified procedure that resulted in incorporation of 10–15% of initial membrane protein compared with 1–5% previously reported. Electrophoretic analysis demonstrated that four out of five erythrocyte membrane proteins were incorporated into the ISCOM, and electron microscopic observations indicated that the ISCOM had a cage-like structure with a diameter of 40 nm, similar to previous ISCOMs. Immunization in the pelvic presacral space (p.s.-p.s.) stimulated significantly higher anti-erythrocyte IgA titres in vaginal fluid than were produced by intraperitoneal (i.p.-i.p.), subcutaneous (s.c.-s.c.), intravaginal (i.vag.-i.vag.), or i.p.-i.vag. immunizations with the same vaccine. Specific IgG titres were less dependent on the route of immunization, with p.s.-p.s., i.p.-i.p. and s.c.-s.c. administration all giving similar high titres while i.p.-i.vag. treatment induced lower titres. These observations using a model ISCOM indicate that mucosal immune responses against membrane proteins were elicited in the female reproductive tract, and that non-mucosal immunization in the pelvis was a more effective route of administration than local application of the ISCOM to the vaginal mucosa.

Keywords: Immunostimulating complex; ISCOM; vagina; mucosal immunity; vaccination; adjuvants; Quil A

INTRODUCTION

Vaccination with a killed or attenuated organism will expose the recipient to genetic materials, irrelevant antigens, and possible toxic components from the micro-organism, but subunit vaccines containing only protective antigens are often less immunogenic. An important method to increase the immunogenicity of many subunit vaccines was provided by the recent development of immunostimulating complexes[1]. Immunostimulating complexes (ISCOMs) are subunit vaccines containing membrane proteins derived from viral envelopes, parasites such as *Toxoplasma gondii*, or eukaryotic cells, incorporated into micelles of the adjuvant Quil A, a saponin derived from the bark of a South American tree, *Quillaia saponaria*[2]. Immunization with ISCOMs causes few side effects and has been shown to produce serum antibody titres ≈10-fold higher than those produced by immunization with an equivalent weight of membrane protein in micellar form or in the form of whole, killed virus particles[1,3–5].

Parenteral immunization with ISCOMs produces effective immunity against many systemic viral infections[1], but little is known about the effects of ISCOM immunization on viral infections at mucosal surfaces, such as *Herpes simplex* 2 and papilloma virus infection in the female genital tract. Immunity at mucosal surfaces generally involves secreted antibody, especially secretory IgA, and local immunization at the mucosal surface is often the optimum way to elicit specific antibody secretion[6]. The only previous study of local, mucosal ISCOM immunization appears to be that of Lovgren[7], who found that two intranasal immunizations of mice with an influenza virus ISCOM produced lower serum titres of IgA and IgG antibodies than comparable subcutaneous administration, and that intragastric administration did not produce a detectable serum response; respiratory tract secretions were not studied. To study the effectiveness of ISCOMs in stimulating

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secretion of specific antibody into mouse vaginal fluid, we prepared a model ISCOM containing sheep erythrocyte membrane proteins and measured anti-erythrocyte IgA and IgG titres in vaginal washings after immunization at parenteral or local, mucosal sites.

MATERIALS AND METHODS

Animals

Outbred female mice of the ICR strain, 60–100 days old, were purchased from Sasco, Inc., Omaha, NE, and were housed adjacent to males of the same strain to induce cycling during vaginal immunization and vaginal fluid collections.

Preparation of ISCOM

Peripheral proteins were removed from 15 mg of sheep erythrocyte membranes (Sigma Chem. Co., St Louis, MO) by incubation in 5 ml 0.1 mM EDTA at pH 11.2 for 15 min at 4°C. The membranes were washed by centrifugation and resuspension twice in 10 mM phosphate buffer at pH 8.0, and integral membrane proteins were then extracted into 3 ml 10 mM Tris buffer at pH 8.0 containing 1% deoxycholate (DOC) and 0.2 mM phenyl methyl sulphonyl fluoride (both from Sigma Chem. Co.). The mixture was sonicated four times for 20 s at 1 min intervals with a Branson sonifier at 30% power (4°C) and stirred for 2 h at room temperature, followed by centrifugation at 100 000*g* for 1 h. The concentration of extracted membrane proteins in the supernatant was ≈ 1.0 mg ml^{-1} (Lowry method with bovine albumin standard). Quil A (Accurate Chem. and Scientific Corp., Westbury, NY) was added to the supernatant at 0.10% w/v, well above the critical micelle concentration of 0.03%[3], and the mixture was incubated overnight at room temperature. The preparation was then dialysed against 0.10% Quil A in Tris buffer to remove DOC and to cause membrane proteins to associate with Quil A micelles. A precipitate formed during dialysis and was removed by centrifugation at 1000*g* for 10 min. The preparation was then subjected to gel filtration on Ultragel AcA 22 (LKB Produkter AB, Bromma, Sweden, retarding proteins smaller than 1200 kDa) to separate Quil A micelles from unbound proteins. Absorbance measurements at 280 nm indicated that essentially all protein applied to the gel eluted in the void volume and was thus associated with micelles. Column fractions containing protein were pooled and lyophilized.

Characterization of ISCOM

The proteins incorporated into the ISCOM were compared to total erythrocyte membrane proteins using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% gels[8]. The ISCOM was also examined by transmission electron microscopy after equal volumes of sample and 2% phosphomolybdate were mixed and applied to grids coated with Formvar/carbon substrates.

Immunization

Five groups of five to six mice each received primary immunization with ISCOM at one of the following sites: subcutaneous (s.c.) near the scapulae, intraperitoneal (i.p.), intravaginal (i.vag.), or in the pelvic presacral space between sacrum and rectum (p.s.). All mice were boosted 4 weeks later at the same site except one group that was primed i.p. and boosted i.vag. I.vag. immunization consisted of inserting a cotton tampon containing 50 μg ISCOM protein in 50 μl PBS into the vagina and changing it daily for 5 days. The mice were housed adjacent to males to induce cycling during i.vag. immunization. All other immunizations involved 10 μg ISCOM protein in 30 μl PBS. The groups are designated s.c.-s.c., i.p.-i.p., i.vag.-i.vag., p.s.-p.s., and i.p.-i.vag.

Vaginal washings

Vaginal washings were obtained by pipetting 50 μl of PBS in and out of the vagina several times. Particulate matter was removed by centrifugation and the supernatants (about 40 μl) were stored at −20°C. For each antibody measurement, vaginal washings were collected once daily for three consecutive days and pooled at the time of use. Vaginal washings were collected from non-immunized mice and pooled for use as controls. The mice were housed adjacent to males to induce cycling during vaginal fluid collection.

ELISA

Sheep erythrocyte membranes (50 μl) at 0.5 mg ml^{-1} in 0.10 M carbonate buffer, pH 9.5, was added to round-bottomed wells of polystyrene microtitre plates (Immulon 1, Dynatech, Alexandria, VA) and centrifuged at 800*g* for 10 min. Following overnight incubation at room temperature in a humid chamber and washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), the plates were incubated sequentially in the following reagents with intermediate washings in PBS-T: 2% calf serum in PBS-T for 30 min, 25 μl of serial 2.25-fold dilutions of vaginal washings in 2% calf serum overnight, 50 μl of 1:2000 dilutions of horseradish peroxidase-conjugated rabbit anti-mouse IgA or IgG (or chain specific, Zymed, South San Francisco, CA) for 2 h, 100 μl of tetramethylbenzidine (Miles Scientific, Naperville, IL) at 0.5 mg ml^{-1} in 0.10 M acetate–citrate buffer, pH 6.0, containing 0.005% H_2O_2 for 1 h, followed by 100 μl 1 M H_2SO_4 to stop the enzyme reaction. The absorbance of plate wells was measured at 450 nm in a Titer Tek microtitre plate reader.

Sample titres are defined as the reciprocal of the highest dilution whose net absorbance (immune vaginal washing minus control vaginal washing) was greater than 0.3. Measurements of the vaginal washing dilutions began with 1/2.25; if net absorbance exceeded 0.3 only in that well the sample had a titre of 2.25^1. If the absorbance was less than 0.3 in that well the sample titre was 2.25^0. An immune vaginal washing standard was included in each measurement. Before titres were calculated, sample absorbances were corrected by the proportion that the standard absorbance on that day differed from its mean over the project period. These adjustments were small, and corrected and uncorrected sample titres did not differ. Geometric mean titres and standard errors were calculated for each immunization group, and the statistical significance of differences between geometric means was determined by *t* tests.

RESULTS

Characterization of ISCOM

The ISCOM had a spherical, cage-like structure with a diameter of 40 nm, and appeared to consist of multiple, small subunits (*Figure 1*). *Figure 2* illustrates the SDS–PAGE electrophoretic pattern of proteins extracted by SDS from washed erythrocyte membranes (column A). Five major protein bands were present (a–e). Column B shows that the same five proteins were present in DOC extracts of erythrocyte membranes. After addition of Quil A to the DOC extract and dialysis to remove DOC, a precipitate formed and was removed by low-speed centrifugation, not sufficient to sediment ISCOMs. Column C shows that the supernatant contained four of the five proteins originally present. An additional minor protein band adjacent to band b may be due to proteolysis during dialysis. Column D shows the protein composition of the ISCOM after unbound proteins were removed by gel filtration. All four available proteins were incorporated into the ISCOM. Measurements of total protein incorporated into the ISCOM compared to total protein extracted from the membranes indicated that 10–15% of extracted protein was incorporated into the ISCOM (three ISCOM preparations, data not shown).

Antibody titres in vaginal fluid

Table 1 shows specific IgA and IgG titres in vaginal fluid after ISCOM immunization by several routes. Mean IgA titres were similar at 4 weeks after primary immunization in all groups except i.vag., where the titre was lower even though the mice received a larger dose of ISCOM. Subsequently, the highest IgA titres were observed in animals immunized in the presacral space (p.s.-p.s.), reaching a maximum $\log_{2.25}$ titre of 5.0 at 7 weeks after priming. This response was significantly higher than that observed in the i.p.-i.p. group at 7 and 10 weeks after priming ($p < 0.05$), but the response in the latter group was well sustained and was nearly equal to that in the p.s.-p.s. group at 14 weeks after priming. In contrast, IgA titres declined after boosting in the s.c.-s.c., i.p.-i.vag., and i.vag.-i.vag. groups, and continued to decline or remained low thereafter. Mean IgG titres at 4 weeks after priming were also similar in the p.s.-p.s., i.p.-i.p., s.c.-s.c., and i.p.-i.vag. groups, but the titre was much lower in the i.vag.-i.vag. group. Subsequently, IgG titres exceeded 8.0 in the p.s.-p.s., i.p.-i.p., and s.c.-s.c. groups until 17 weeks, while they declined to intermediate values in the i.p.-i.vag. group and remained low in the i.vag.-i.vag. group.

After the last vaginal washings were collected the immunization sites of all mice were examined for evidence of tissue reactions. One mouse in the i.p.-i.p. group had a small adhesion of the duodenum to the posterior abdominal wall, and four out of six mice in the presacral group had a white, opaque mass that appeared to be unabsorbed vaccine at the injection site. No other abnormalities were observed.

Figure 1 Electron micrograph of Quil A ISCOM negatively stained with phosphomolybdate. The ISCOM has a spherical, cage-like structure with a diameter of 40 nm, and appears to consist of multiple, small subunits. Membrane proteins are thought to be oriented in the Quil A micelles much as they are in intact membranes, with their hydrophobic domains bound to Quil A in the interior of the ISCOM while their hydrophilic portions are exposed to the aqueous medium at the surface (× 130 000)

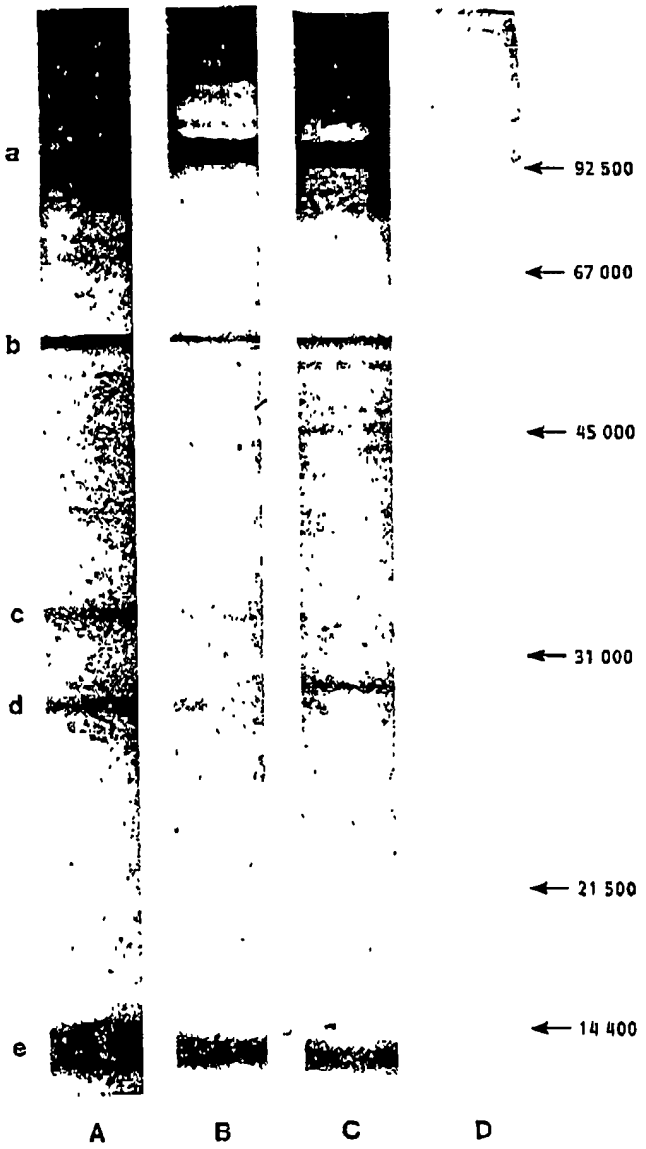

Figure 2 Electrophoretic pattern of sheep erythrocyte membrane proteins in sodium dodecyl sulphate 10% polyacrylamide gel. The five major protein bands are designated a–e. Columns A–D are described in the text. The positions of standard proteins with known molecular weights are shown on the right

Table 1 Log_{2n} geometric mean IgA and IgG antibody titres in vaginal washings after ISCOM immunization

Route of immunization	Isotype	Time after primary immunization (weeks)*				
		4	7	10	14	17
s.c.-s.c.	A	3.8±0.5	3.0±1.3	2.8±0.9	1.0±0.4	
	G	7.2±0.3	>8.0	>8.0	>8.0	7.7±0.3
i.p.-i.p.	A	6.2±0.9	2.8±0.5	3.0±0.5	3.6±0.5	
	G	8.0±0.4	>8.0	>8.0	>8.0	9.0±0.4
p.s.-p.s.	A	4.5±0.5	5.0±1.1	4.7±0.6	3.8±1.5	
	G	7.7±0.4	>8.0	>8.0	>8.0	7.5±0.4
i.p.-i.vag.	A	4.2±0.5	1.5±0.5	1.7±0.3	1.5±0.6	
	G	6.0±0.3	5.5±0.9	5.5±0.7	5.5±0.7	3.5±1.0
i.vag.-i.vag.	A	1.8±0.3	0.7±0.4	0.0	0.7±0.3	-
	G	0.8±0.8	1.3±0.9	0.2±0.2	1.0±0.4	-

*All mice received booster immunization 4 weeks after priming

DISCUSSION

A model immunostimulating complex containing Quil A and sheep erythrocyte membrane proteins was prepared for this study by modification of a previously described procedure[9]. The modified procedure used a dialysable detergent, DOC, to solubilize membrane proteins instead of Triton X-100 followed by dialysis to remove DOC and gel filtration to separate the ISCOM from unbound protein. The original procedure removed detergent and purified the ISCOM in one step, sucrose gradient ultracentrifugation, but required electron microscopic examination of gradient fractions to locate the purified ISCOM. The modified procedure was simpler to perform, produced an ISCOM with a spherical, cage-like structure similar to that of previously described ISCOMs[1], and resulted in 10–15% of initial membrane protein being incorporated into the ISCOM compared with 1–5% previously reported[9]. Four out of five erythrocyte membrane proteins were incorporated into the ISCOM, suggesting that the modified procedure may be suitable for many membrane proteins.

The magnitude and isotype of secretory immune responses in vaginal fluid after ISCOM immunization were influenced by the route of immunization. The highest IgA titres were observed after non-mucosal immunization in the pelvic presacral space. This is an intramuscular site involving muscles attached to the ventral surface of the sacrum and controlling tail movements. IgA titres in vaginal fluid after p.s.-p.s. immunization were five or more times higher than after s.c.-s.c. immunization near the scapulae with the same vaccine. The IgG titres in these groups were quite similar, suggesting that the different IgA titres were not simply due to differences in the overall intensity of immune responses. The immunological basis of relatively high IgA titres in vaginal fluid after parenteral ISCOM immunization in the presacral space is unknown, but comparable results have been obtained using horse ferritin as antigen[10]. A route of immunization that elicits relatively high IgA titres in vaginal fluid is of particular interest because IgA has been shown to be more protective than IgG against bacterial infection in the intestine[11] and respiratory tract[12]. Local application of a larger dose of ISCOM to the vaginal mucosa via tampons was ineffective in producing either an IgA or IgG response in vaginal fluid, either in the i.vag.-i.vag or the i.p.-i.vag. regimens. It is unlikely that the large doses of i.vag. vaccine caused tolerance or suppression because in comparable studies using horse ferritin as antigen we have observed that large i.vag. antigen doses gave relatively weak immune responses while smaller antigen doses gave even smaller responses. In order for intravaginal antigen to elicit an immune response it must reach lymphoid cells or lymphatic vessels in vaginal mucosa, and it is possible that the ISCOM, with a diameter of 40 nm, was too large to penetrate the stratified squamous epithelium of the vagina. The route of ISCOM immunization had less effect on IgG responses in vaginal fluid, with s.c.-s.c., i.p.-i.p., and p.s.-p.s. immunization all producing similar high titres.

These results indicate that a model ISCOM containing erythrocyte membrane proteins was easily prepared and caused little or no tissue toxicity in any of the immunization sites we used. It elicited a secretory immune response in vaginal fluid that included a significant IgA component when it was administered in the presacral space. These observations suggest that further work with an ISCOM containing envelope proteins from herpes or papilloma viruses would be of interest[13].

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